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13. ABSTRACT *(Maximum 200 Words)*

An essential requirement in understanding how genes contribute to genetic disease is the thorough knowledge of the mechanisms controlling gene expression at the level of transcription. In this project we are identifying and functionally characterizing the NF1 regulatory region, and evaluating the molecular mechanisms that regulate NF1 transcription during normal cellular differentiation and the events leading to NF1-related neoplasia. Overlapping regions of the NF1 proximal promoter have been cloned using a series of DNA fragments created by PCR and characterized for use in the luciferase assays to isolate specific binding motifs near the NF1 transcription start site. Luciferase reporter assays have identified a 425 bp region displaying activities 50 to 80 higher than baseline reporter levels. Mutations at putative CRE and SP1 binding sites immediately 5' to the transcription start site have dramatic effects that lead to a 70-90% decrease in reporter activity. As well, these assays have revealed a putative repressor region within the NF1 promoter region corresponding to CCCTC rich sequences between the transcription and translation start sites. Preliminary gelshift assays confirm binding of SP1 and CRE to their putative recognition sequences and provide the first evidence identifying functional sites likely involved in regulating NF1 transcription.

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Transcriptional regulation and targeting of NF1 gene expression

Introduction

Our understanding of neurofibromatosis has increased significantly since the identification of the NF1 gene ten years ago. Primarily, these breakthroughs have increased our understanding of how the neurofibromin protein works in cells, and how mutations in the NF1 gene result in the clinical symptoms that make up the disease (Shen etal. 1998; Guha 1998). In contrast, surprisingly little work has been undertaken to identify and characterize how the NF1 gene is regulated at the level of transcription (Mancini etal. 1999). An essential requirement to understand how genes contribute to genetic disease is the thorough knowledge of the mechanisms controlling gene expression at the level of transcription. ***Our goal in this project*** is to characterize the NF1 regulatory region, and to evaluate the molecular mechanisms that regulate NF1 transcription during normal cellular differentiation and the events leading to NF1-related tumours. This project is in the process of identifying and functionally characterizing the *NF1* regulatory region, as well as evaluating the molecular mechanisms that regulate *NF1* transcription during normal cellular differentiation and the events leading to *NF1*-related neoplasia.

Body of Report:

Our research strategy for the past year, as outlined in our revised *Statement of Work*, is presented in the context of these specific research tasks.

Task 1. (a) Cloning of the NF1 promoter region
(months 1-6) (b) Preparation of NF1 luciferase reporter constructs

Task 2. (c) Transfections of reporter constructs
(months 3-15) (d) Manipulation of intracellular cAMP levels in transfected cultured cells
(e) PCR mutagenesis of CREB site and other factor binding sites

Task 1a. Cloning of *NF1* promoter sequence and reporter construction:

Our first step was to clone overlapping regions of the *NF1* proximal promoter and to test these regions in a luciferase reporter system. Two approaches, one involving long range sequences and a second involving sequences in the immediate vicinity of the NF1 transcription start site were used.

I. PCR amplification and cloning: The process of cloning specific NF1 promoter regions by PCR, generating the luciferase constructs and confirming their sequences has taken up the bulk of our time in the past year. Initially I hired post-doctoral fellow Dr Tim C. Groves who worked on the cloning tasks for two months, prior to his leaving for a position in industry. Later in the spring of 2001, post-doctoral fellow Dr Min-Xu Zou joined the project and has been training in my lab and working on the project since that time. DNA amplifications used PCR primers specific to human *NF1* sequences retrieved from Genbank (HSU 17084) and TIGR (#281489; see

Figure 1A; Hajra etal 1994). We were also able to retrieve working draft sequence from the Human Genome Database (AC027793; 148kb) that contained a 16 kb fragment in which we located the NF1 transcription start site with approximately 9 kb of 5' UTR. Five separate PCR primers were designed and tested.

DRNF1	5'- ACAGCCTCCCCAGGAGATTAGCGG - 3'
DRNF2	5'- CTGGCACTCGCCAGCTGAGCCCAG - 3'
DRNF3	5'- GGAAGTGGGGATCCTTTCCACGGCC - 3'
DRNF4	5'- TCGTCGAAGCGGCTGACCACGGCC - 3'
DRNF5	5'- GAGCTATGATTGAGCCACTGCACTCC - 3'

We have successfully amplified five separate regions in the CpG rich *NF1* promoter region corresponding to sequences encompassing primers 2-3 (259 bp), 1-3 (426 bp), 2-4 (693 bp), 1-4 (810 bp) and 5-3 (1522 bp; **Figure 1**). These amplified products were sequenced and compared favourably with the various Genbank sequences. Particularly problematic for us though, was the GC rich region between nts positions 3601-3821 (Genbank HSU17084) that contains CCCTC repeat sequences and proved to be difficult to amplify consistently. Therefore our primer design strategy took this into account and we used primer DRNF3 as a boundary site to delineate this repeat sequence. **Figure 2** shows the relative locations of the PCR primers within the NF1 promoter region. The resulting PCR templates were ligated into the TOPO-TA cloning vector (Invitrogen), transformed and then screened. Clones positive for the *NF1* insert were purified by alkaline lysis and sequenced at the core DNA sequencing facility here at the University of Western Ontario to ensure sequence fidelity prior to cloning into the PGL3 luciferase reporters. In several cases, PCR primers were designed with *Xho* I and *Hind* III restriction sites included at their 5' ends to allow for cloning directly into the PGL3 luciferase expression vectors.

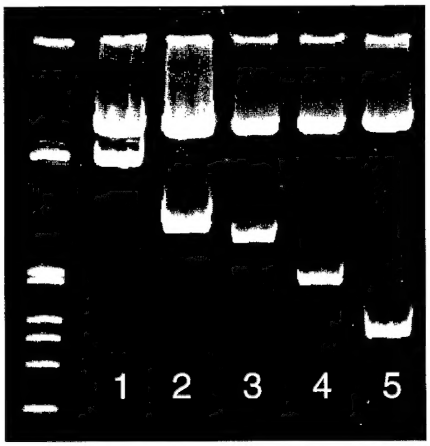

Figure 1. Acrylamide gel showing RE digests of PCR products amplified from the NF1 promoter region and cloned into the pGL3 luciferase expression vector.

Lane 1: fragment 5-3 (1522 bp)
Lane 2: fragment 1-4 (810 bp)
Lane 3: fragment 2-4 (693 bp)
Lane 4: fragment 1-3 (426 bp)
Lane 5: fragment 2-3 (259 bp)

Amplified *NF1* fragments were mini-prep'd and ligated in a 1:1 molar ratio into the pGL3-Basic Luciferase reporter vector (Promega) for 16 hours at 16°C. These ligations subsequently were transformed in bacterial hosts and the resulting clones were screened and sequenced to confirm the DNA sequences.

II. Isolation of NF1 sequences from BAC clones: At the beginning of our research we also employed an alternate strategy to capture the NF1 promoter region for cloning and to characterize distal NF1 regulatory elements. This

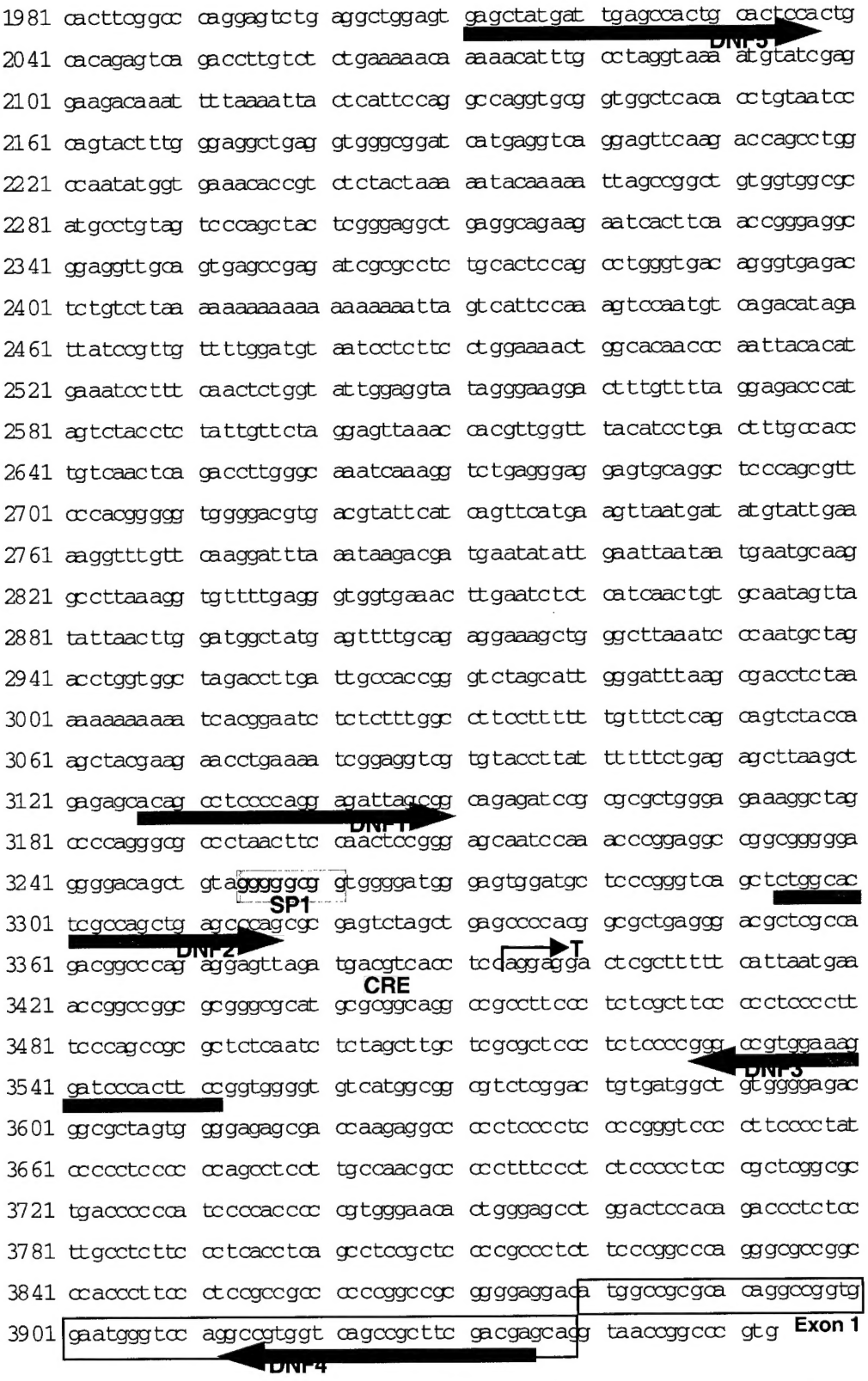

Figure 2: NF1 5'UTR sequence (HSU17084)

strategy was chosen as previously described, because our group and others (personal communication) have experienced difficulties in the past in consistently amplifying across the very GC-rich sub-regions within the NF1 promoter region. We used the 426 bp NF-13 fragment of the NF1 regulatory region to screen a human BAC RPCI-11 library from the MRC Genome Resource Facility (Centre for Applied Genomics, Hospital for Sick Children, Toronto). Twelve NF1 positive clones have been isolated from that BAC library and are presently being characterized in my lab. Four of these BAC clones were grown up in liquid culture and BAC DNA was isolated and purified from two of these clones. Our analyses of the Genbank DNA sequences and restriction digest mapping of the BACs allowed us to predict the presence of a 4.5kb BamH1 fragment containing the NF1 promoter region (**Figure 3**). We are undertaking experiments at the present time to confirm this 4.5 kb region, which we have cloned into the TOPO-TA cloning vector (Invitrogen). Since our primary strategy using PCR amplification has succeeded in cloning subregions of the NF1 promoter in the vicinity of the transcription start site, we will now limit our ongoing experiments to the 4.5 kb fragment from these BamH1 clones. In this way, we can determine the existence of more distal regions of the NF1 5'-UTR and generate several other relevant reporter constructs.

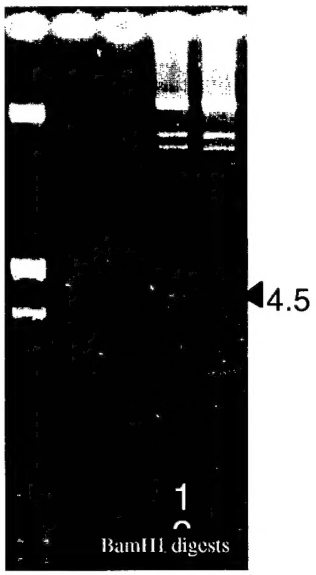

Figure 3. Agarose gel BamH1 digest of Human BAC Clones revealing the 4.5 kb fragment that includes the NF1 promoter region (lanes 1,2)

Task 1b. Preparation of NF1 luciferase reporter constructs

Our strategy has been to focus on regions surrounding the transcription start site and to specifically target putative transcription factor binding motifs such as CRE and SP1 (Hajra et al. 1991; Mancini et al. 1998). To date, nine overlapping luciferase reporter constructs have been created which differ by the presence or absence of specific 5' and 3' sub-sequences (**Figure 4**).

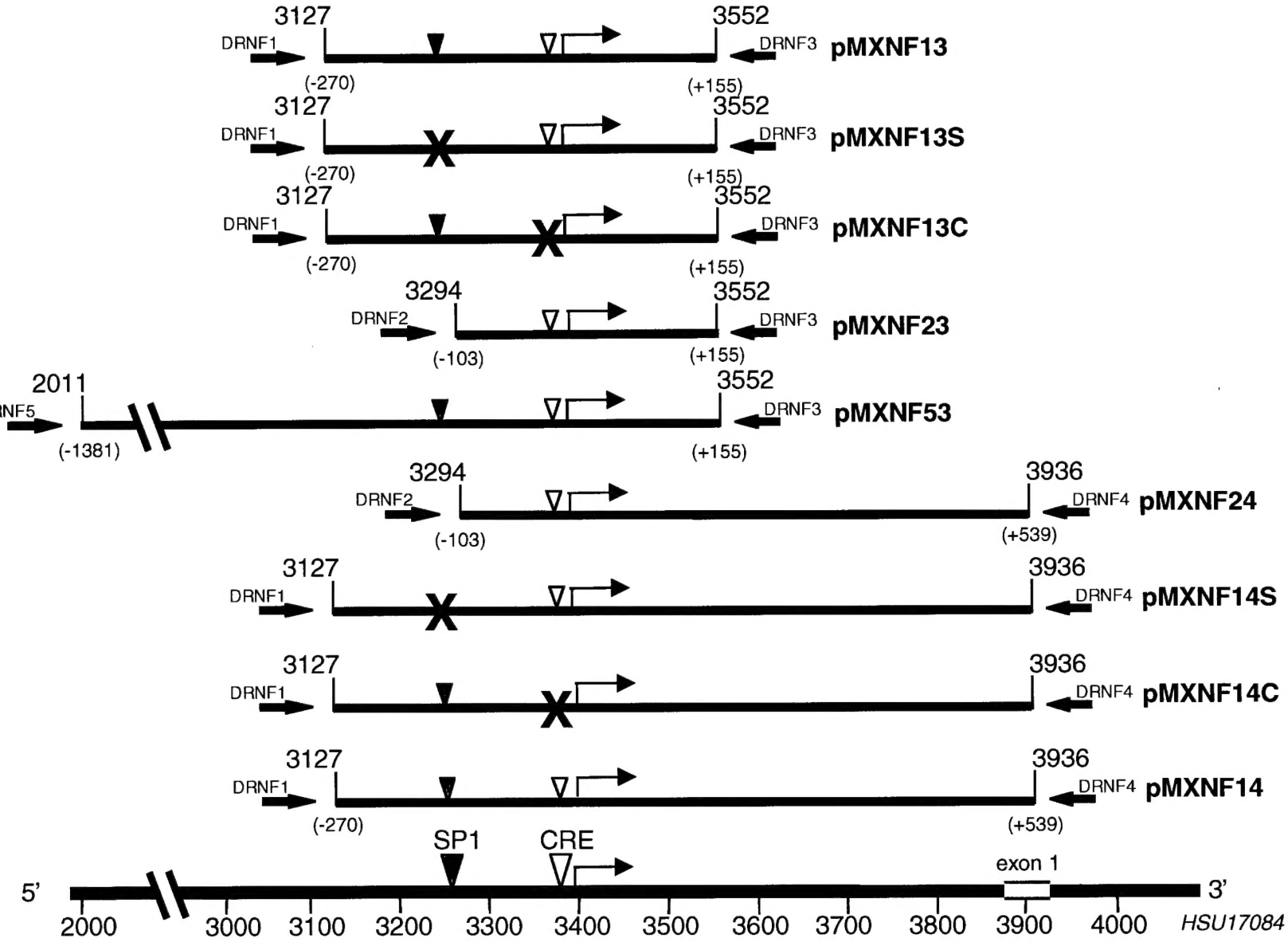

Figure 4: NF1 promoter constructs

Task 2c. Transfection of NF1 luciferase reporter constructs

Human HeLa cells and mouse embryonic fibroblast 293 cells were seeded in each well of 6-well 35 mm^2 tissue culture plates. Transfections were carried out in multiple sets in triplicate (three separate transfections for each experimental condition, performed on the same day) using 4 µl of lipofectamine reagent (Gibco-BRL) and 1 µg of the reporter plasmids (Mancini etal. 1999). Cells were harvested 48 hours after transfection and cell lysates were assayed for luciferase activity using the protocols from the Promega luciferase kit. Total protein was determined by Bradford assay, while activities were standardized relative to pGL3-Basic vector controls. We confirmed equivalent transfection efficiencies for the pGL3 vectors by cotransfecting with the pSV-β galactosidase vector and determining β-Gal activities in the cell lysates (data not shown).

Table 1 (see Appendix) shows results of pGL3 – NF1 reporter construct activities in luciferase expression experiments performed in triplicate under the same conditions. Background luciferase activities (relative light units /µg protein: RLU/mg protein) were determined for the pGL3-Basic vector and with pGL3-SV40 as a positive control. Luciferase activities for all constructs are shown, relative to the activities seen in the pGL3-basic experiments (i.e. X times pGL3). Results from experiments using constructs containing site-directed mutations at CpGs within the CRE and SP1 factor binding sites are also shown (i.e. -14s, -14c; -13s, -13c). We found that we were able to maximally drive expression of the luciferase reporter with a 425 bp fragment (pMXNF-13) containing intact sequences flanking the putative NF1 transcription start site. Reporter activities with PMXNF-13 ranged from 49-87 fold increases relative to the pGL3-basic reporter. Other constructs showed variable activities that apparently depended on the presence of other specific DNA sequences. For example, the activity of pMXNF-14 was substantially (5 fold) less than PMXNF-13, apparently due to the presence in this construct of 384 bp containing repetitive CCCTC sequences positioned 3' to the start site.

The comparisons of all reporter activities relative to PMXNF-13 (**Table 2**) and PMXNF-14 (**Table 3;** also in Appendix) show consistency across experiments. Particularly interesting are the effects seen when the putative CRE and SP1 motifs have been mutated. In these experiments, we used a PCR-based approach to perform site-directed mutagenesis at the CRE or the SP1 motifs, changing the central CpG dinucleotide to a TpG dinucleotide (CRE: TGA**CG**TCA →TGA**TG**TCA; SP1: GGGG**CG**GG →GGGG**TG**GG). The mutagenised PCR products were ligated into the pGL3 luciferase reporter vector, the presence of the appropriate mutation(s) was verified by sequencing, and the vector was transfected as previously described. Direct comparison between the PMXNF-13 and reporters PMXNF-13s and –13c show up to a 90% decrease in activity when the CRE (60-70% decrease) or the SP1 sites (70-90% decrease) are mutated. These transient transfection experiments are very supportive of a functional role for these particular transcription factors in regulating NF1 gene expression. As a result we have begun experiments to stably transfect pMXNF-13 into recipient cells. These experiments will allow us to determine in vivo effects on NF1 expression in cells manipulated in culture.

Task 2d. Manipulation of intracellular cAMP levels in transfected culture cells.

These experiments have begun, in light of the recent transfection experiments with the pMXNF-13 and –13c constructs. We have recently received several appropriate cultured neuroblastoma cell lines from ATCC. Two of these lines (HCN2 and HTB186) differ dramatically in their levels of cAMP responsiveness and will be particularly useful for these experiments to be completed over the next 3 months.

Task 2e. Mutagenesis of CREB site and other factor binding sites

This task involves the transfection experiments described earlier (Tables 1-3) using the luciferase reporters possessing the altered CRE and SP1 sites and as described in detail in the previous section. As well, preliminary electrophoretic mobility shift assays (EMSAs; gel shifts) have been undertaken to characterize the functional specificity of the CRE and SP1 motifs identified within the NF1 promoter. These gelshift experiments involve oligonucleotides targeted to these CRE and Sp1 motifs as well as oligonucleotides in which we have introduced point mutations at the CpGs contained with these motifs. Nuclear extracts have been prepared from cultured cells and incubated with annealed oligonucleotides containing the normal / mutant CRE or Sp1 binding sites. The double-stranded oligonucleotides were endlabelled with [γ^{-32}P] ATP using T4 polynucleotide kinase and labelled to a specific activity of 1 - 2 x 10^5 cpm / pmol. Representative results are shown in **Figure 5**. For SP1, a distinct set of proteins is bound to the oligonucleotide corresponding to the normal SP1 sequence located 140 bps upstream of the start site (figure 5b) and we were able to identify one band within this complex that reacted with the anti-SP1 antibody (5a; lane 3). In contrast, mutating the SP1 recognition sequence (lanes 4-6) dramatically inhibited binding of this protein complex. Similar results were seen with the CRE oligonucleotide, where one prominent protein would not bind to the oligonucleotide in which we had included a CG to TG mutation. These in vitro experiments provide further evidence that the SP1 and CRE motifs are viable motifs that likely play a role in regulating NF1 expression. Further work is commencing, as we begin DNA footprinting experiments across the NF1 promoter region.

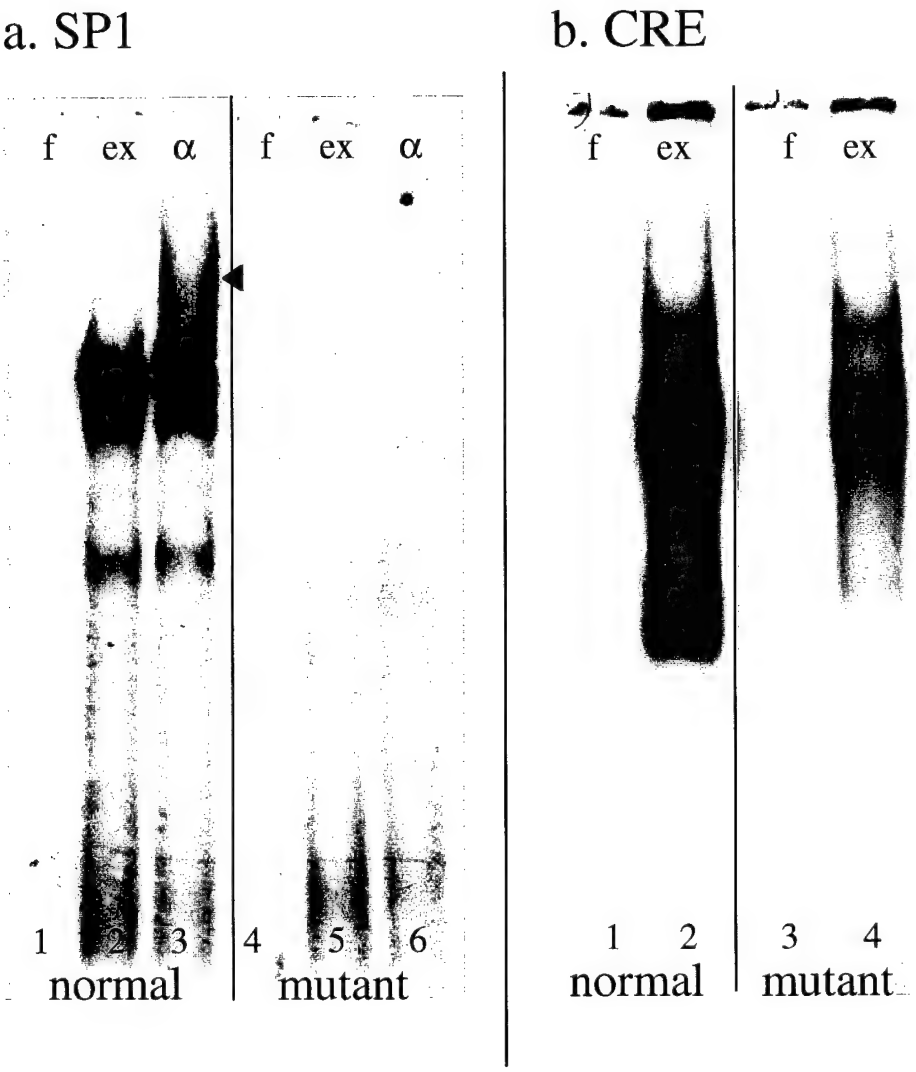

Figure 5. Gelshift assays using oligos specific to (a) SP1 and (b) CRE. In each experiment, oligos possessing altered (mutant) binding motifs were also used. These CG to TG alterations dramatically changed the protein binding profiles seen binding to the SP1 and the CRE sequences. The arrowhead indicates the supershift seen in the presence of the anti-SP1 antibody (Santa Cruz). F: free probe, no protein; ex: probe and cell extract; α: probe, cell extract and anibody.

Key Research Accomplishments

- Overlapping regions of the NF1 proximal promoter have been cloned using a series of DNA fragments created by PCR and characterized for use in the luciferase reporter assays.
- Luciferase reporter assays have identified a 425 bp region displaying activities up to 80 fold higher than baseline reporter levels.
- Mutations at putative CRE and SP1 binding sites immediately 5' to the transcription start site have dramatic effects that lead to a 70-90% decrease in reporter activity.
- These assays have revealed a putative repressor region within the NF1 promoter region corresponding to CCCTC rich sequences between the transcription and translation start sites.
- Preliminary gelshift assays confirm binding of SP1 and CRE to their putative recognition sequences and provide the first evidence identifying functional sites likely involved in regulating NF1 transcription.

Reportable Outcomes:

- Manuscript is being prepared.

Conclusions:

- A major component lacking in our knowledge of the biology of the NF1 gene is the mechanism by which cells regulate *NF1* transcription.
- This study is beginning to provide a clearer understanding of the functional sites within the NF1 regulatory region that are responsible for regulation of NF1 gene expression. CRE and Sp1 elements are the first of these putative regulatory elements we have identified.
- Since the CRE motif is the target binding site for the CRE binding protein (CREB; reviewed in Andrisani 1999), complex transcriptional activation by CREB suggest possible roles for NF1 in propagating diverse cellular responses, including apoptosis, cell proliferation, and neuronal signalling.
- As well, given the apparent interactions between NF1 isoforms and cAMP second messengers our work provides additional evidence that the involvement of the cyclic AMP transduction pathway in oligodendrocyte development which may have a complimentary role in regulation of NF1 gene expression (Fieber, 1998; Gutmann et al. 1993).

References:

Andrisani OM (1999) CREB-mediated transcriptional control. Crit Rev Eukaryot Gene Expr 9(1):19-32.

Fieber LA (1998) Ionic currents in normal and neurofibromatosis type 1-affected human Schwann cells: induction of tumor cell K current in normal Schwann cells by cyclic AMP. J Neurosci Res 54(4):495-506.

Guha A (1998) Ras activation in astrocytomas and neurofibromas. Can J Neurol Sci 25(4):267-81.

Gutmann DH, Tennekoon GI, Cole JL, Collins FS, Rutkowski JL (1993) Modulation of the neurofibromatosis type 1 gene product, neurofibromin, during Schwann cell differentiation. J Neurosci Res 36(2):216-23

Hajra A, Martin-Gallardo A, Tarle SA, Freedman M, Wilson-Gunn S, Bernards A, Collins FS (1994) DNA sequences in the promoter region of the NF1 gene are highly conserved between human and mouse. Genomics 21(3):649-52.

Mancini DN, Singh SM, Archer TK, Rodenhiser DI (1999). Site-specific DNA methylation in the neurofibromatosis (NF1) promoter interferes with binding of CREB and SP1 transcription factors. Oncogene18(28):4108-19.

Mancini DiNardo, Butcher D, Robinson D, Archer T, and Rodenhiser D *(PI)*. (2001) Functional analysis of CpG methylation in the BRCA1 promoter region. Oncogene 20: 5331-5340.

Shen MH, Harper PS, Upadhyaya M (1996) Molecular genetics of neurofibromatosis type 1 (NF1). J Med Genet 33(1):2-17.

Appendix:

Table 1: Representative results showing pGL3 – NF1 reporter construct activities in luciferase expression experiments.

Table 2: pGL3 – NF1 reporter luciferase expression: luciferase activities relative to pMXNF-14.

Table 3: pGL3 – NF1 reporter luciferase expression: luciferase activities relative to pMXNF-13.

Table 1: Representative results showing pGL3 – NF1 reporter construct activities in luciferase expression experiments.

cell line	Construct	(nt Number)	RLU / μg prot	X times PGL3 activity
HeLa	pGL3-SV40	-	318354	-
	PGL3-Basic	-	382	1
	pMXNF -53	-1381 to +539	24199	63
	pMXNF -14	-270 to +539	4906	13
	pMXNF -13	-270 to +155	33446	87
	pMXNF -23	-103 to +155	18025	47
	pMXNF -24	-103 to +539	5905	15
	pMXNF –14s	-270 to +539 SP1-	6944	18
	pMXNF –14c	-270 to +539 CRE-	4043	11
	pMXNF –13s	-270 to +155 SP1-	4607	12
	pMXNF –13c	-270 to +155 CRE-	10008	26
293	PGL3-SV40	-	382506	-
	PGL3-Basic	-	2764	1
	pMXNF -53	-1381 to +539	60963	22
	pMXNF -14	-270 to +539	26212	9
	pMXNF -13	-270 to +155	134985	49
	pMXNF -23	-103 to +155	100046	36
	pMXNF -24	-103 to +539	24741	9
	pMXNF –14s	-270 to +539 SP1-	10116	4
	pMXNF –14c	-270 to +539 CRE-	10656	4
	pMXNF –13s	-270 to +155 SP1-	41576	15
	pMXNF –13c	-270 to +155 CRE-	50032	18

Luciferase experiments were performed in triplicate under the same conditions. Background luciferase activities (relative light units /μg protein: RLU/mg protein) were determined for the pGL3-Basic vector and with pGL3-SV40 as a positive control. Luciferase activities for all constructs are shown, relative to the activities seen in the pGL3-basic experiments (i.e. X times pGL3). Results from experiments using constructs containing site-directed mutations at CpGs within the CRE and SP1 factor binding sites are also shown (i.e. -14s, -14c; -13s, -13c).

Table 2

pGL3 – NF1 reporter luciferase expression: luciferase activities relative to pMXNF-14.

cell line	Construct	(nt Number)	set 1	set 2	set 3
HeLa	pGL3-SV40	-	50	24	65
	PGL3-Basic	-	0.03	0.02	0.07
	pMXNF -14	**-270 to +539**	**1 X**	**1 X**	**1 X**
	pMXNF -53	-1381 to +539	-	-	5
	pMXNF -13	-270 to +155	3	4	4
	pMXNF -23	-103 to +155	2.4	3	4
	pMXNF -24	-103 to +539	0.5	0.4	1.2
	pMXNF –14s	-270 to +539 SP1-	-	0.3	1.4
	pMXNF –14c	-270 to +539 CRE-	-	0.3	0.8
	pMXNF –13s	-270 to +155 SP1-	-	-	0.9
	pMXNF –13c	-270 to +155 CRE-	-	-	2
293	pGL3-SV40	-	9	4	15
	PGL3-Basic	-	0.001	0.08	0.1
	pMXNF -14	**-270 to +539**	**1 X**	**1 X**	**1 X**
	pMXNF -53	-1381 to +539	-	-	2
	pMXNF -13	-270 to +155	4	3	5
	pMXNF -23	-103 to +155	4	3	4
	pMXNF -24	-103 to +539	1	1	1
	pMXNF –14s	-270 to +539 SP1-	-	0.8	0.4
	pMXNF –14c	-270 to +539 CRE-	-	1	0.4
	pMXNF –13s	-270 to +155 SP1-	-	-	1.6
	pMXNF –13c	-270 to +155 CRE-	-	-	2

Luciferase experiments were performed in triplicate on each of the same days (i.e. set1, set2 and set3; total of nine repeats) under the same conditions. Background luciferase activities (relative light units /μg protein: RLU/mg protein) were determined for the pGL3-Basic vector and with pGL3-SV40 as a positive control. Luciferase activities for all constructs are shown, relative to the activities seen in the pMXNF14 constructs. Results from experiments using constructs containing site-directed mutations at CpGs within the CRE and SP1 factor binding sites are also shown (i.e. -14s, -14c; -13s, -13c).

Table 3

pGL3 – NF1 reporter luciferase expression: luciferase activities relative to pMXNF-13.

cell line	Construct	(nt Number)	set 1	set 2	set 3
HeLa	**pMXNF-13**	**-270 to +155**	**1 X**	**1 X**	**1 X**
	pMXNF-14	-270 to +539	0.3	0.2	0.1
	pMXNF-53	-1381 to +539	-	-	0.7
	pMXNF-23	-103 to +155	0.7	0.7	0.5
	pMXNF-24	-103 to +539	0.2	0.1	0.2
	pMXNF–14s	-270 to +539 SP1-	-	0.1	0.2
	pMXNF–14c	-270 to +539 CRE-	-	0.1	0.1
	pMXNF–13s	-270 to +155 SP1-	-	-	0.1
	pMXNF–13c	-270 to +155 CRE-	-	-	0.3
293	**pMXNF-13**	**-270 to +155**	**1 X**	**1 X**	**1 X**
	pMXNF-14	-270 to +539	0.3	0.3	0.2
	pMXNF-53	-1381 to +539	-	-	0.4
	pMXNF-23	-103 to +155	0.8	0.9	0.7
	pMXNF-24	-103 to +539	0.3	0.4	0.2
	pMXNF–14s	-270 to +539 SP1-	-	0.3	0.1
	pMXNF–14c	-270 to +539 CRE-	-	0.4	0.1
	pMXNF–13s	-270 to +155 SP1-	-	-	0.3
	pMXNF–13c	-270 to +155 CRE-	-	-	0.4

Luciferase experiments were performed in triplicate on each of the same days (i.e. set1, set2 and set3; total of nine repeats) under the same conditions. Background luciferase activities (relative light units /μg protein: RLU/mg protein) were determined for the pGL3-Basic vector and with pGL3-SV40 as a positive control. Luciferase activities for all constructs are shown, relative to the activities seen in the pMXNF13 constructs. Results from experiments using constructs containing site-directed mutations at CpGs within the CRE and SP1 factor binding sites are also shown (i.e. -14s, -14c; -13s, -13c).